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Fibronectin Modulates the Effects of HIV-1 Tat on the Growth of Murine Kaposi's Sarcoma-Like Cells Through the Down-Regulation of Tyrosine Phosphorylation

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HIV-1 Tat plays a role in the pathogenesis of Kaposi's sarcoma. We therefore investigated the effect of Tat on the growth of murine Kaposi's sarcoma-like spindle (TTB) cells derived from dermal lesions. We observed that Tat and a peptide corresponding to the carboxyl-terminal region (Tat_{65-80}) containing an RGD sequence inhibit TTB cell proliferation only when cells are cultured on fibronectin. This inhibitory effect correlates with redistribution of the α_v integrin subunit on the surface of TTB cells and with down-regulation of tyrosine phosphorylation of specific substrates due to an increased tyrosine phosphatase activity. Indeed, phenylarsine oxide, a potent inhibitor of phosphotyrosine phosphatases, prevented the effects of Tat on TTB cells. We therefore argue that the action of Tat on TTB cells is mediated by the RGD motif through an integrin-based cell signaling pathway involving the activity of phosphotyrosine phosphatase(s), which would lead to a decrease in the levels of phosphotyrosine-containing proteins, among which is erk-2/$p42^{MAPK}$. *(Am J Pathol 1998, 152:1599–1605)*

The HIV-1-Tat protein transactivates viral and cellular genes of HIV-1-infected cells.[1] In addition, Tat can be released by infected cells[2] and acts extracellularly in the microenvironment regulating the functions of mesenchymal and immunocompetent cells, because Tat binds to cell surface receptors and mimics the action of growth factors and other molecules in modulating signal cascades.[3,4] Indeed, Tat binds to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins[5] and activates cellular genes.[6,7] In vascular endothelial cells, Tat has been shown to specifically bind and activate the Flk-1/kinase insert domain receptor (Flk/kdr),[8] a vascular endothelial growth factor (VEGF)-A tyrosine kinase receptor, which is a major regulator of vasculogenesis and angiogenesis.[9] The finding that Tat binds to Flk/kdr may explain its angiogenic activity.[10] Indeed, Tat has been postulated to have a role in the pathogenesis of Kaposi's sarcoma (KS) because it is angiogenic and it enhances the proliferative effects of basic fibroblast growth factor (bFGF).[11] Moreover, *tat* transgenic mice develop KS-like lesions.[12] Although a γ-herpes virus is considered the etiological agent of sporadic and epidemic KS,[13] KS in AIDS patients is a more aggressive disease when compared with sporadic KS; the angiogenic properties of Tat have been proposed to enhance KS tumor formation.

Here we studied the effect of synthetic Tat on KS-like murine spindle TTB cells,[14] and we show that Tat-fibronectin interactions modulate TTB cell proliferation and signal transduction.

Materials and Methods

Materials

Synthetic Tat was kindly provided by P.G. Fassina (Tecnogen, Caserta, Italy). The activity of the synthetic protein has been described elsewhere.[15] The Tat peptide amino acids 65 to 80; (Tat_{65-80}) was purchased from Intracel (Cambridge, MA). Peptides GRGDS and GRYDS were purchased from Sigma Chemical Co. (St. Louis, MO). The 15-amino-acid peptide W, corresponding to the signal peptide of HIV-1 gp 160, rabbit anti-α_v polyclonal antibody, and rabbit serum against the β_1 integrin subunit were kindly provided by M. Tattanelli, G. Tarone, and R. Pardi, respectively. The anti-erk-2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

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Cell Lines

TTB cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek, Israel). TTB cells, which show a spindle phenotype, co-express markers specific for endothelial, smooth muscle, and antigen-presenting cells.[14] In addition, these cells grow in response to serum and some angiogenic molecules such as fibroblast growth factors and hepatocyte growth factor (Z. Wu and J.A.M. Maier, unpublished results).

Immunofluorescence Staining

Cells were seeded on glass coverslips either uncoated or coated with human plasma fibronectin. When subconfluent, cells were washed and fixed in phosphate-buffered saline (PBS), pH 7.6, containing 3% (w/v) paraformaldehyde and 2% (w/v) sucrose. After washing, cells were permeabilized as previously described[16] before incubation with primary antibodies. Secondary antibodies were tetramethylrhodamine isothiocyanate (TRITC)-abeled swine immunoglobulins against rabbit IgG (Dakopatts, Glostrup, Denmark). Cells were routinely counterstained with fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) to visualize F-actin.

Cell Growth Assays

Cells were seeded in 24-well plates (Costar, Cambridge, MA), either uncoated or coated with fibronectin. Tat was added every 24 hours for three times in medium containing 10% FCS. In some experiments, Tat_{65-80} was used instead of full-length Tat, whereas peptide W was used as its negative control. Control cells were treated with the same buffer used to dilute Tat (PBS, 10 mmol/L dithiothreitol, 1 mg/ml bovine serum albumin). In another set of experiments, cells were exposed to phenylarsine oxide (PAO; 50 μmol/L) with or without Tat. At the end of the treatment, cells were trypsinized and counted by trypan blue exclusion.

Cell Surface Biotinylation

TTB cells were seeded into either uncoated or fibronectin-coated 60-mm dishes and incubated at 37°C for increasing times. At each time point, cells were washed six times in buffer A (25 mmol/L Hepes, pH 7.4, 138 mmol/L NaCl, 1.3 mmol/L $CaCl_2$, 0.4 mmol/L $MgSO_4$, 5 mmol/L KCl, 5.6 mmol/L D-glucose). Cells were incubated twice for 20 minutes at 4°C with 0.5 mg/ml Sulfo-NHS-biotin (Pierce, Rockford, IL), diluted in buffer A. After extensive washing, cells were lysed in lysis buffer (50 mmol/L Tris/HCl, pH 8.5, 150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.05 U/ml aprotinin). Cell extracts were then immunoprecipitated with a rabbit polyclonal antibody against the α_v integrin subunit. The immune complexes were resolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and electroblotted onto nitrocellulose (Millipore, Bedford, MA). The blots were incubated with peroxidase-conjugated streptavidin (Amersham, Little Chalfont, UK) diluted 1:2000. Biotinylated proteins were detected with the SuperSignal chemiluminescence kit (Pierce).

Immunoprecipitation and Western Blot

TTB cells were washed, lysed in ice-cold lysis buffer, and centrifuged. After preclearing with preimmune serum, lysates were immunoprecipitated with the anti-phosphotyrosine monoclonal antibody PY20 (Santa Cruz Biotechnology). The immunocomplexes were bound to protein G-Sepharose and eluted in Laemmli buffer at 95°C for 5 minutes. Samples were resolved by SDS-PAGE, transferred to nitrocellulose sheets at 150 mA for 16 hours, and probed with a polyclonal anti-phosphotyrosine antibody (provided by T. Maciag, Rockville, MD). Secondary antibodies were labeled with horseradish peroxidase (Pierce). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins. As activated erk-2/$p42^{MAPK}$ is phosphorylated also on tyrosine residues, we detected erk-2/$p42^{MAPK}$ by immunoprecipitating cell lysates with PY20. Western blot was performed using a rabbit polyclonal anti-erk-2/$p42^{MAPK}$ antibody.

Tyrosine Phosphatase Activity

After exposure to Tat (10 ng/ml) for 30 minutes, TTB cells were washed and lysed in ice-cold lysis buffer. Tyrosine phosphatase activity was determined using a photometric enzyme immunoassay according to the manufacturer's instruction (Boehringer Mannheim, Mannheim, Germany) and expressed as $(T_0 - T_n)/T_0$, where T_0 is the optical reading of the control to which both proteins and an excess of orthovanadate were added and T_n is the optical reading at a certain time as described.[17] Reactions have been verified to be linear with respect to time and quantity of protein.

Results

Tat-Fibronectin Interactions Modulate the Growth of TTB Cells

The effect of synthetic Tat on the growth of Kaposi-like spindle TTB cells was studied in cells seeded onto either uncoated or fibronectin-coated culture wells. Fibronectin does not modulate TTB cell growth (not shown). We then treated TTB cells for 72 hours with increasing concentrations of synthetic Tat, and viable cells were counted. As shown in Figure 1, 1 to 100 ng/ml Tat had no effect when added to cells cultured without any substrate. However, when TTB cells were seeded in fibronectin-coated wells, Tat inhibited cell proliferation in a dose-dependent fashion. The effect was maximal when cells were exposed to 10 ng/ml Tat, a concentration that is similar to that de-

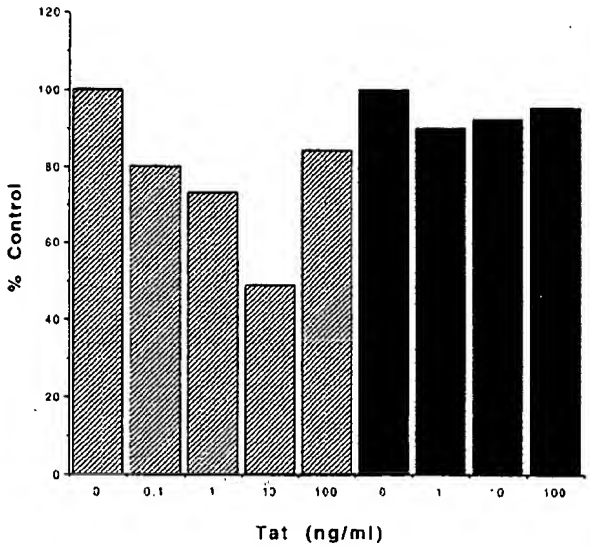

Figure 1. Effect of exogenous Tat on the growth rate of TTB cells cultured on fibronectin. TTB cells were seeded onto either uncoated (**solid bars**) or fibronectin-coated (**hatched bars**) wells. Cells were then treated with synthetic Tat at the indicated concentrations for 72 hours as described in Materials and Methods. At the end of the treatment, cells were trypsinized and counted by trypan blue exclusion. Each point is the mean of three experiments, each performed in triplicate. Data are expressed as percentage of the control. Standard deviation never exceeded ±6%.

tected in the sera of HIV-1-positive individuals.[3] The same phenomenon was observec treating fibronectin-adherent TTB cells with recombinant Tat from two different sources; on the contrary, no inhibitory effect was observed when TTB cells plated on gelatin-coated wells were exposed to Tat (not shown). These data are in agreement with our previous results showing that human endothelial cells were growth inhibited by Tat only when grown on fibronectin.[18] In contrast with our results, Tat was reported to stimulate the proliferation of human AIDS-KS spindle cells.[19] This discrepancy could be due to the different experimental conditions used; human spindle cells were cultured in the absence of fibronectin in a conditioned medium derived from HTLV-II-infected T cells[20] containing a variety of cytokines altering cell growth and synergizing with Tat in the induction of cell proliferation. Moreover, TTB phenotype is quite different from that of AIDS-KS spindle cells, as TTB cells co-express markers typical of endothelial cells, smooth muscle cells, and macrophages.[14] It has been shown that Tat interacts with the integrin receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$.[5] As the inhibition of TTB cell proliferation by Tat was dependent on cell adhesion to fibronectin, we verified whether adhesion to fibronectin modulated the expression and/or surface exposure of integrin receptors in TTB cells. By immunofluorescence, clusters of α_v-containing integrins were detectable at focal contacts in cells cultured on gelatin or on plastic (Figure 2B), whereas α_v was no longer detectable upon TTB cell adhesion to fibronectin within 4 hours from seeding and assumed a widespread pattern (Figure 2D). On the contrary, adhesion of TTB cells to either fibronectin (Figure 2H) or gelatin (not shown) did not affect the expression or the subcellular distribution of β_1. However, only the distribution and not the total amount of α_v integrins at the cell surface changed dramatically on fibronectin; indeed, culture on fibronectin did not alter the total amount of α_v integrins localized at the cell surface (Figure 3).

Tat interacts with integrins through the RGD motif.[5] This interaction mediates different effects of Tat on AIDS-KS and vascular cells, such as adhesion, induction of growth, migration, invasion, and angiogenesis.[19] We therefore verified whether Tat-mediated inhibition of TTB cell proliferation on fibronectin could depend on the RGD sequence contained in the carboxyl-terminal region of Tat.[21] The effects of equimolar amounts of synthetic Tat and of a peptide corresponding to the carboxyl-terminal sequence of Tat (Tat_{65-80}) containing the RGD domain were compared. As shown in Figure 4, Tat_{65-80} was as effective as Tat in inhibiting TTB cell proliferation upon adhesion to fibronectin. In contrast, the irrelevant 15-amino-acid peptide W showed no effect on TTB cell proliferation. Moreover, a 200-fold molar excess of peptide GRGDS almost completely reversed the effect of Tat, whereas the control peptide GRYDS had no effect (Figure 4). When added in the absence of Tat, both RGD- and RYD-containing peptides had no effect on TTB cell proliferation (Figure 4).

Inhibition of TTB Cell Growth Correlates with Down-Regulation of Tyrosine Phosphorylation of Specific Substrates

As protein tyrosine phosphorylation is implicated in the control of cell growth,[22] the possibility that Tat inhibited tyrosine phosphorylation of various substrates in TTB cells was tested by detecting phosphotyrosine-containing proteins. Tat (10 ng/ml) decreased the tyrosine phosphorylation of three proteins with an apparent molecular weight of 140, 100, and 80 kd only when cells were grown on fibronectin-coated dishes (Figure 5A). Interestingly, the concentration of Tat necessary to down-regulate tyrosine phosphorylation of the three proteins was identical to that necessary to inhibit the growth of TTB cells. The inhibition of tyrosine phosphorylation of the three aforementioned proteins was maximal after 30 minutes and returned to the baseline levels within 1 hour (Figure 5B). With a longer exposure of the filters, we could observe decreased phosphotyrosine levels also in protein(s) with an apparent molecular weight of 40 kd (not shown).

As the level of tyrosine phosphorylation is the result of the balance between activation of protein phosphotyrosine kinases and phosphatases, we evaluated whether Tat activated phosphotyrosine phosphatase(s) in TTB cells. Indeed, we observed an increase of tyrosine phosphatase activity in cell extracts derived from TTB cells treated with Tat (10 ng/ml) for 30 minutes (Figure 6A). Interestingly, the decrease in tyrosine phosphorylation that follows treatment with Tat could be prevented by the addition to the cell culture of PAO (50 μmol/L), a specific inhibitor of tyrosine phosphatase activity[23] (Figure 6B). To show that the action of Tat on TTB cell growth involves the activity of tyrosine phosphatase(s), we cultured TTB cells in the presence of PAO and Tat. As shown in Figure

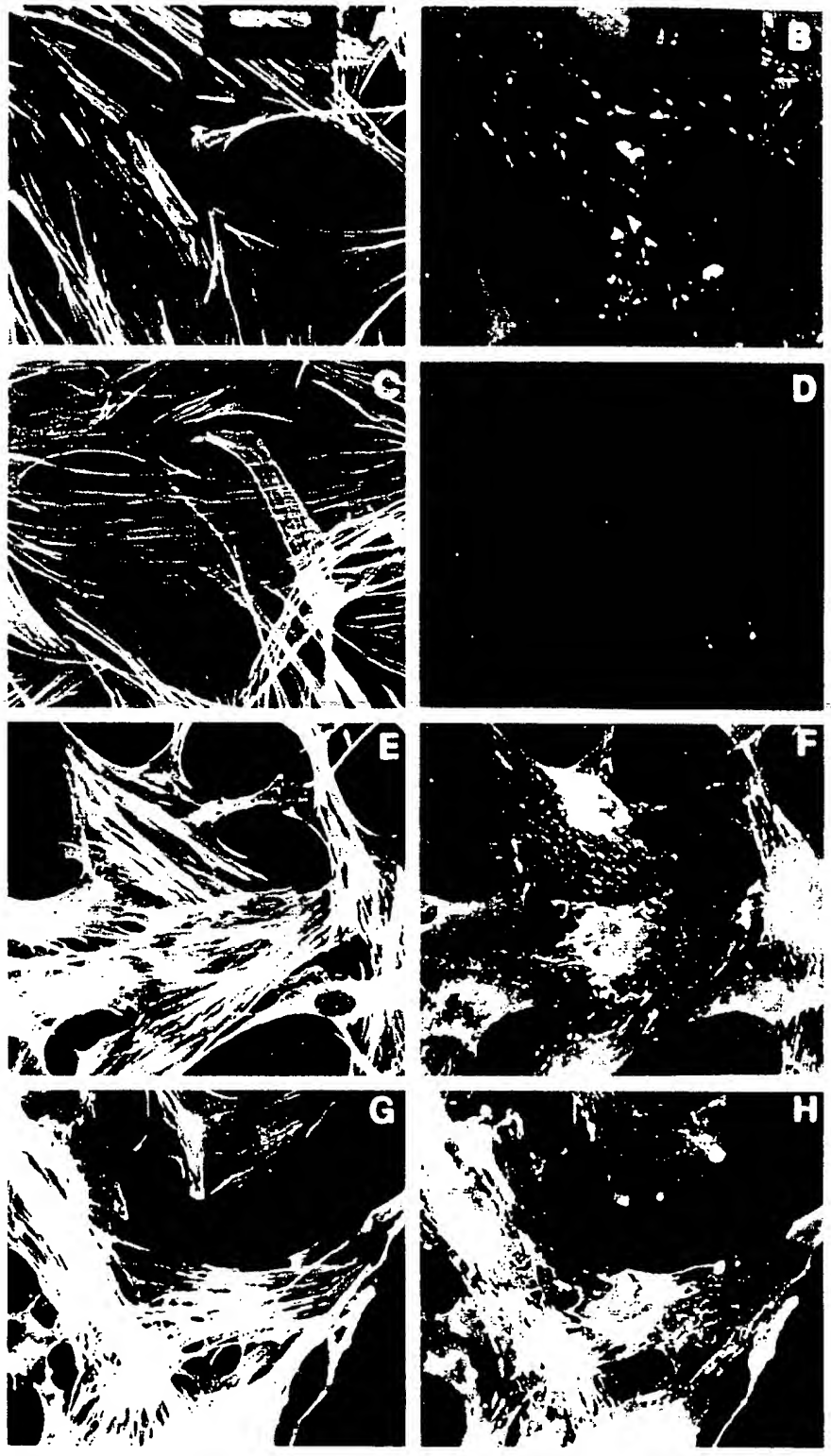

Figure 2. Modulation of integrin surface exposure upon adhesion of TTB cells to fibronectin. TTB cells were cultured for 48 hours on either uncoated (A, B, E, and F) or fibronectin-coated (C, D, G, and H) coverslips. Cells were then fixed and stained with either rabbit anti-α_5 (B and D) or rabbit anti-β_1 (F and H) polyclonal antibody, followed by a TRITC-labeled swine anti-rabbit IgG polyclonal antibody. Cells were counterstained with FITC-labeled phalloidin (A, C, E, and G).

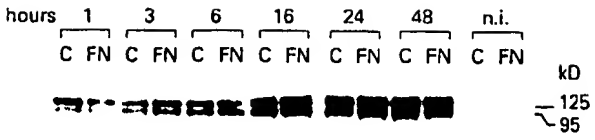

Figure 3. Expression of cell surface α_v upon TTB cell adhesion to fibronectin. TTB cells were cultured for increasing times on either uncoated or fibronectin-coated dishes. Membrane proteins were biotinylated and cell extracts were immunoprecipitated with anti-α_v antibody. After electroblot, biotinylated proteins were detected with peroxidase-conjugated streptavidin. C, control cells cultured on uncoated dishes; FN, cells cultured on fibronectin-coated dishes. Lanes 1 to 12, extracts immunoprecipitated with anti-α_v; n.i., extracts of cells cultured for 48 hours immunoprecipitated with rabbit non-immune IgG.

6C, in the presence of PAO, Tat did not inhibit TTB cell proliferation. Similar results were obtained using Tat_{65-80} (not shown).

Inhibition of TTB Cell Growth Correlates with Down-Regulation of Tyrosine Phosphorylation of erk-2/p42^{MAPK}

Previous findings have indicated a role of erk-2/p42^{MAPK} in the growth of human KS spindle cells.[24] Moreover, a recent report shows the activation of the MAP kinase pathway in cells of the central nervous system.[25] Erk-2/p42^{MAPK} activation occurs by way of a kinase cascade that includes one or more MAP kinase kinases that induce phosphorylation of threonine and tyrosine residues.[26] Therefore, we evaluated the levels of tyrosine-phosphorylated erk-2/p42^{MAPK} in Tat-treated TTB cells

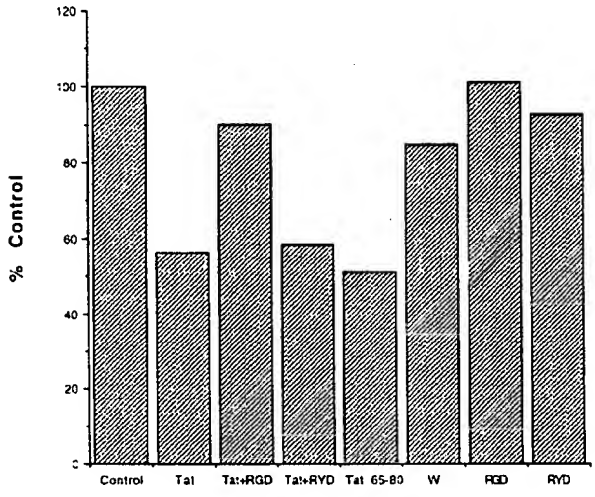

Figure 4. Role of RGD sequence in Tat-mediated inhibition of TTB cell growth. TTB cells were plated on fibronectin-coated wells and treated with 10 ng/ml synthetic Tat, either in the absence or in the presence of a 200-fold molar excess of GRGDS (referred to as RGD) or GRYDS (referred to as RYD). In another set of experiments, fibronectin-adherent TTB cells were treated with 0.7 nmol/L Tat_{65-80} peptide (equimolar to 10 ng/ml Tat) or control peptide W. Each point is the mean of three experiments, each performed in triplicate. Data are expressed as percentage of the control. Standard deviation never exceeded ±6%.

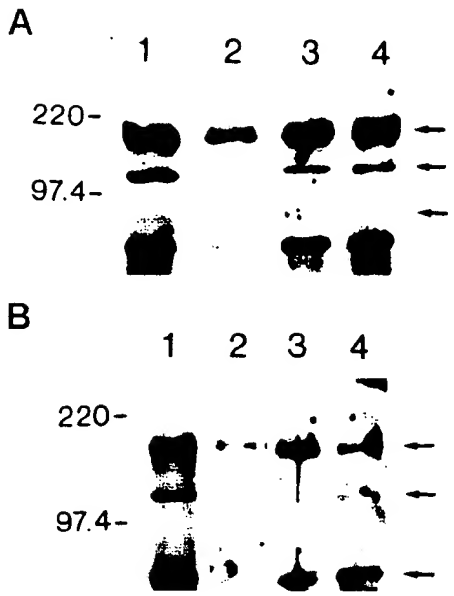

Figure 5. Effects of Tat on tyrosine phosphorylation in TTB cells. Cells were treated with Tat as described in Materials and Methods. Cell lysates (300 μg of protein) were immunoprecipitated with a monoclonal antibody and immunoblotted with a polyclonal antibody against phosphotyrosine as described in Materials and Methods. A: Lanes 1 and 2, cells cultured on fibronectin, either untreated or treated with Tat (10 ng/ml), respectively; lanes 3 and 4, cells cultured on plastic, either untreated or treated with Tat (10 ng/ml), respectively. B: Lane 1, untreated cells; lanes 2 to 4, TTB cells treated with Tat (10 ng/ml) for 30 minutes and 1 and 4 hours.

grown on fibronectin. Figure 7 shows that the levels of tyrosine-phosphorylated erk-2/p42^{MAPK} were rather high in control cells. Within 30 minutes, Tat (10 ng/ml) markedly down-regulated the levels of tyrosine-phosphorylated erk-2/p42^{MAPK}. Again, treatment with PAO restored the levels of tyrosine-phosphorylated erk-2/p42^{MAPK} and inhibited the dephosphorylating effect of Tat (Figure 7).

Discussion

The signals for proliferation, differentiation, and survival of anchorage-dependent cells are provided by extracellular growth factors and matrix and are mediatec by a variety of kinases that are often regulated by their level of tyrosine phosphorylation.[27] Under physiological conditions, the protein tyrosine phosphorylation levels in a cell are maintained by phosphotyrosine kinases and phosphatases.[27] There is evidence that negative signals from phosphotyrosine phosphatases may be responsible for growth arrest. Here we show that, upon adhesion to fibronectin, Tat inhibits TTB cell proliferation through a mechanism that down-regulates phosphotyrosine levels. In particular, we demonstrate that Tat induces the dephosphorylation on tyrosine residues of erk-2/p42^{MAPK}, thus inhibiting the activity of this kinase that plays a pivotal role in integrating and amplifying signals from different extracellular stimuli, including growth- and differentiation-promoting agents.[28] Our results are in agreement with a previous report showing an intimate relationship between the proliferation of human KS spindle cells

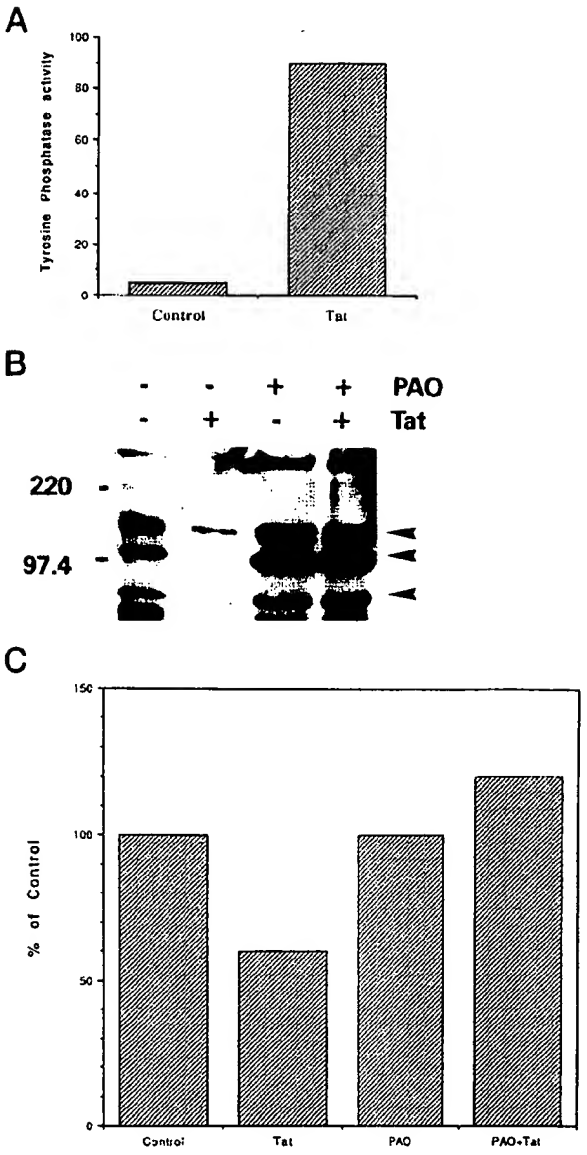

Figure 6. Role of phosphotyrosine phosphatase in mediating Tat activity in TTB cells. A: TTB cells grown on fibronectin were treated with Tat (10 ng/ml) for 30 minutes or left untreated. Tyrosine phosphatase activity was quantitated in 30 μg of proteins by a photometric enzyme immunoassay as described in Materials and Methods. Each point is the mean of two experiments performed in triplicate. B TTB cells grown on fibronectin were treated with PAO (50 μmol/L) for 30 minutes before adding Tat (10 ng/ml) or left untreated. Cell lysates (300 μg of protein) were immunoprecipitated with a monoclonal antibody and immunoblotted with a polyclonal antibody against phosphotyrosine as described in Materials and Methods. Lanes 1 and 2, untreated and Tat-treated cells; lanes 3 and 4, cells exposed to PAO only and to PAO and Tat, respectively. C: TTB cells were plated on fibronectin-coated wells and treated with 10 ng/ml synthetic Tat, either in the absence or in the presence of PAO (50 μmol/L). After 3 days, cells were counted as described above. Data are expressed as percentage of the control. Standard deviation never exceeded ±6%.

and MAP kinase activation.[23] Recently, MAP kinase has been shown to be activated by Tat in cells of the central nervous system; however, this phenomenon has not been linked to any functional event in neurons or in glial cells, although it is argued that MAP kinase activation may cause dysfunctions leading to the AIDS dementia complex.[25]

In our system, Tat activates phosphotyrosine phosphatase(s). Accordingly, PAO, a specific inhibitor of phosphotyrosine phosphatases,[23] prevented the effects of Tat in TTB cells; in PAO-treated cells, Tat did not inhibit cell growth and tyrosine phosphorylation of various substrates, including erk-2/p42^{MAPK}. Therefore, we argue that cellular responses to Tat may result from direct activation of phosphotyrosine phosphatase(s). A few authenticated instances indicate that activation of a phosphotyrosine phosphatase is the primary event in signal transduction; among others, increased intracellular calcium activates phosphotyrosine phosphatase 2B, which is essential for T cell activation.[27] In addition, it should be mentioned that a novel phosphotyrosine phosphatase has been isolated from Tat-treated vascular cells by RNA fingerprinting (M. Mariotti and J.A.M. Maier, unpublished), suggesting that phosphotyrosine phosphatases have a role in mediating the effects of Tat on cellular activities.

The role of fibronectin in modulating Tat action on TTB cells is intriguing. As Tat binds to cell surface integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, we argue that Tat action on TTB is mediated through the binding of the RGD motif of Tat to integrins, as supported by the fact that Tat_{65-80} mimicked the action of Tat. As adhesion of TTB to fibronectin correlated with loss of the typical patterned localization of α_v at focal contacts, we argue that Tat may not bind to $\alpha_v\beta_3$ when clustered at focal contacts; on the contrary, when $\alpha_v\beta_3$ clusters are disrupted after cell adhesion to fibronectin, cell surface $\alpha_v\beta_3$ might be available for Tat binding, with consequent activation of an intracellular cascade of events leading to the down-regulation of tyrosine phosphorylation of various substrates. Among these, erk-2/p42^{MAPK} dephosphorylation would result in the inhibition of cell proliferation. It is worth noting that phosphotyrosine phosphatases have recently been implicated in the regulation of integrin-dependent signal transduction. A direct role for CD45 in ICAM-3-mediated lymphocyte aggregation was demonstrated.[29] More generally, reduction of cell adhesion is associated with an induction of phosphotyrosine phosphatase activity.[30] Ad-

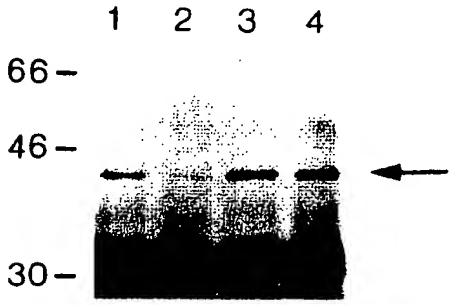

Figure 7. Effects of Tat on erk-2/p42^{MAPK}. TTB cells were plated on fibronectin-coated wells and treated with 10 ng/ml synthetic Tat, either in the absence or in the presence of PAO (50 μmol/L). Cell extracts were immunoprecipitated with the anti-phosphotyrosine PY20 antibody, and Western blot was performed using a polyclonal antibody against erk-2/p42^{MAPK}. Lanes 1 and 2, untreated and Tat-treated cells; lanes 3 and 4, cells exposed to PAO only and to PAO and Tat, respectively.

ditional studies are required to identify the individual specific phosphotyrosine phosphatase(s) involved in $\alpha_v\beta_3$ signaling in Tat-treated TTB cells.

Acknowledgments

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